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# Larval masquerade: a new species of paedomorphic salamander (Caudata: Plethodontidae: *Eurycea*) from the Ouachita Mountains of North America

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## Abstract

Species with truncated developmental patterns may go undetected if they resemble the juveniles of their close relatives. Herein we present an example of this phenomenon with the description of a highly divergent, relict species of stream-dwelling plethodontid salamander from the Ouachita Mountains of North America. Both mitochondrial and nuclear sequence data show that this new species is most closely related to its syntopic relative, *Eurycea multiplicata*. Interestingly, *E. multiplicata* exhibits the ancestral biphasic (metamorphic) life cycle, whereas the new species maintains an aquatic larval form throughout life (paedomorphic) and superficially resembles larval *E. multiplicata*. The new species is the first known paedomorphic plethodontid from the Ouachita Mountains, and the most divergent paedomorphic salamander discovered in over seventy years. This species represents an independent instance of the evolution of paedomorphosis associated with a porous streambed, which may facilitate vertical seasonal movements. This new species currently has an extremely limited known distribution and is of immediate conservation concern.

**Key words:** endemic species, *Eurycea*, life history

## Introduction

Developmental truncations can produce species that resemble the larvae or juveniles of close relatives (e.g. Camp *et al.* 2002). However, some ontogenetic stages, such as larval forms, can be difficult to identify morphologically and are often understudied (Thomas *et al.* 2005; Vences *et al.* 2005; Barber & Boyce 2006; Hubert *et al.* 2010; Ko *et al*. 2013). Therefore, without close examination of specific life history stages, species that arise from developmental truncations can go undetected, resulting in an underestimation of biodiversity.

The lungless salamanders, Family Plethodontidae, have diversified extensively since the Late Cretaceous (Vieites *et al.* 2007), and are the most species rich clade of caudates (currently 440 recognized species; AmphibiaWeb 2014). More than 80% of recognized plethodontids have been described since Dunn's (1926) seminal work on the family. This is in part due to increased survey efforts, but also due to the application of molecular techniques, which have revealed many cryptic species in morphologically conserved groups (e.g. Highton 1989; Chippindale *et al.* 2000; Garcia-Paris *et al.* 2000; Jockusch *et al*. 2002). Plethodontids are known to exhibit a wide diversity of life histories and developmental modes (Wake 1966; Chippindale *et al.* 2004; Mueller *et al*. 2004; Bonett *et al*. 2014), but ecologically and developmentally distinct new species are extremely rare, especially within North America, which has been extensively surveyed (Dunn 1926; Highton 1989; Camp *et al.* 2009).

The plethodontid tribe Spelerpini includes five genera (*Eurycea, Gyrinophilus, Pseudotriton, Stereochilus,* and *Urspelerpes*) and thirty-five recognized species. These primarily stream-dwelling salamanders display multiple areas of endemism in the southern Appalachian Mountains, Edwards Plateau, and Interior Highlands (Ouachita Mountains and Ozark Plateau; Fig. 1). While most spelerpines have a biphasic (metamorphic) life history, typical of amphibians, several members of this clade display paedomorphosis (Wake 1966; Ryan & Bruce 2000; Bonett *et*

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*al.* 2014), the phenomenon where adults retain ancestral juvenile characteristics throughout life (e.g. gills, tailfin, larval throat structure; Duellman & Trueb 1986). Paedomorphosis in spelerpines has likely evolved independently in the Ozark Plateau, Edwards Plateau, Cumberland Plateau, and Gulf Coastal Plain (Wake 1966; Bonett & Chippindale 2004; Niemiller *et al.* 2008; Bonett *et al.* 2014). However, despite the Ouachita Mountains being a major biogeographic feature of eastern North America, the only spelerpine known to occur there is the widespread metamorphic *Eurycea multiplicata.*

The taxonomy of *E. multiplicata* and related species (*E. tynerensis* and *E. spelaea*) has been historically misconstrued by life history. Until recently, the alternative life histories of *E. tynerensis* within the Ozarks were considered to be two distinct species. Paedomorphic populations in the Ozarks were classified as *E. tynerensis*, and metamorphic populations were considered to be a subspecies of *E. multiplicata* (*E. m. griseogaster*; Petranka 1998). However, genetic analyses strongly support the monophyly of *E. tynerensis* and *E. m. griseogaster*, and *E. m. multiplicata* from the Ouachita Mountains as sister to the Ozarkian species: *E. tynerensis* (including *E. m. griseogaster*) and *E. spelaea* (Bonett & Chippindale 2004). Therefore, life history is variable within *E. tynerensis* in the Ozarks, but paedomorphosis has never been reported in *E. multiplicata*, in the Ouachita Mountains.

Here we describe a new species of paedomorphic salamander from the Ouachita Mountains. This new species is morphologically, genetically, ecologically, and developmentally distinct from syntopic *Eurycea multiplicata*, with phylogenetic reconstruction placing it as sister to *E. multiplicata*. This discovery represents the most divergent paedomorphic salamander described in the past 70 years (Moore & Hughes 1939; Carr 1939). This new species appears to have an extremely limited distribution and should be of immediate conservation concern.

## Material and methods

The first specimen of the new species was discovered on 23-May 2011 (by MAS and KJI), while collecting *Eurycea multiplicata* at Lake Catherine State Park, Hot Spring County, Arkansas, USA (Fig. 1). This specimen was originally identified as highly distinct from *E. multiplicata*, based on sequence data, and the presence of very small immature egg follicles in a relatively large larval specimen. The original sampling area and nearby vicinity were searched several times in 2011 and 2012. It was not until February 2013 that additional specimens were found in the original location and an adjacent stream. These specimens allowed us to confirm that the new species is paedomorphic, and based on putative morphological characteristics was easily distinguishable from syntopic larvae of metamorphic *Eurycea multiplicata*. Our description is based upon genetic comparisons to representative samples of *E. multiplicata* from throughout the Ouachita Mountains (as well as outgroups; Table 1 & 2), and morphological comparisons to syntopic larvae of *E. multiplicata*. The species description is supported under the General Lineage, Phylogenetic, and Biological Species Concepts (Mayr 1942; de Queiroz 2007).

**Morphological Analyses**. Twenty-four specimens of the new species and 12 specimens of syntopic *E. multiplicata* larvae were collected from 25-March to 20-April 2013, and returned alive to the University of Tulsa. Specimens were anesthetized in a 0.1% solution of tricaine methanesulfonate (MS-222) for morphometric measurements. Specimens were handled in accordance with Institutional Animal Care and Use Committee (IACUC; TU-0029).

Dorsal and ventral images of the body and head were taken with a Pentax K-7 (14.6 megapixel), or Canon G9 (12.1 megapixel) camera. ImageJ (Abramoff *et al.* 2004) was used to obtain the following measurements: snout-vent length (SVL), trunk length (TL), head length (HL), head width (HW), interorbital distance (ID), eye to nostril distance (EN), eye diameter (ED), trunk width posterior to forelimbs (TRW) and tail width posterior to hindlimbs (TW). All standard morphological measurements were made to the nearest 0.1mm. Analysis of Covariance (ANCOVA) in SPSS v17, with SVL as the covariate, were used to test if any of the measurements were significantly different between the new species and syntopic larval *E. multiplicata*. Several specimens were fixed in 10% buffered formalin, including the holotype, and a subset were cleared and stained with alcian blue and alizarin red for cartilage and bone respectively (Hanken & Wassersug 1981). The osteology of seven specimens of the new species were compared to ten *E. multiplicata* larvae.

For geometric morphometrics, a series of 11 landmarks on body images, and eight landmarks and 35 semi-landmarks on head images were used to capture the shape of each specimen (Fig. 2). Images were rescaled and superimposed using procrustes superimposition on CoodGen7a (Sheets 2003). Head and body shape were then

analyzed using principle component analysis (PCA) with the program PCAGen7a (Sheets, 2003). We also tested for significant morphological shape variation between the two species, employing the Goodall's F-test in TwoGroup7 (Sheets 2003). To account for potential differences in ontogeny (size), we also compared eight of the smallest specimens of the new species to the *E. multiplicata* larvae. We realize that we are still comparing juvenile larvae to subadults (or small adults) of the new species, but to date, juvenile specimens of the new species have not been found.

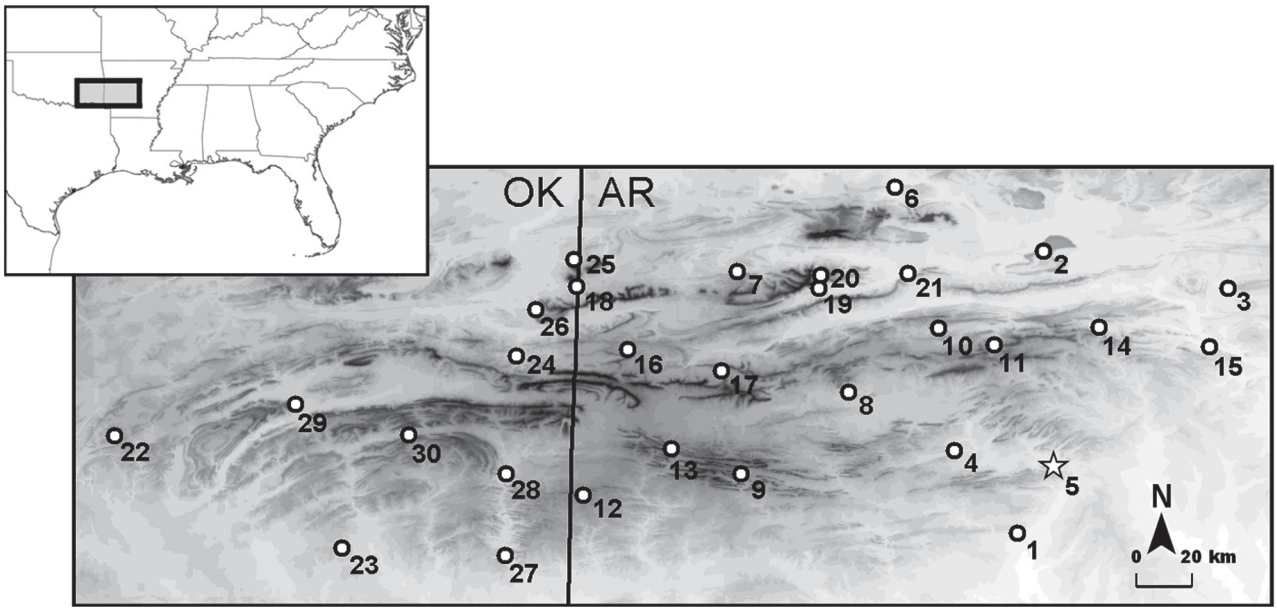


**FIGURE 1.** Distribution map of sampling localities across the Ouachita Mountains. The open star indicates the type locality and only known site of *E. subfluvicola*, where *E. multiplicata* were also sampled. Open circles indicate other genetic sampling localities for *E. multiplicata.* The numbers correspond with those in Table 1. Shaded box on inset map of the southeastern United States shows location of the Ouachita Mountains. AR=Arkansas and OK=Oklahoma.

**DNA extraction, PCR, sequencing, and phylogenetic analyses.** Genomic DNA was extracted from tail tips or liver tissue using QIAGEN DNeasy extraction kits. Portions of the mitochonial gene *cytochrome b* (*Cytb*), and the nuclear recombination-activating gene 1 (*Rag1*), were amplified via polymerase chain reaction (PCR) using gene specific primers and standard conditions (Arévalo *et al.* 1994; Moritz *et al.* 1992; Timpe *et al.* 2009). Successful PCR products were cleaned using EXOSAP-IT (USB Corp.). Sequencing reactions were performed using BigDye v3.1 (Applied Biosystems Inc.), unincorporated dye terminators were removed with Sephadex G-50 (Sigma). Reaction products were sequenced in both directions on an ABI 3130xl capillary sequencer. For *Cytb,* we sequenced 19 individuals of the new species, and 40 individuals of *Eurycea multiplicata*, representing the breadth of genetic diversity of this taxon across the Ouachita Mountains (Bonett & Chippindale 2004; Steffen & Bonett in prep.). Thirteen *E. multiplicata* were from Lake Catherine State Park, 12 of which were collected syntopically with the new species (Table 1). For the more conserved gene *Rag1* we sequenced 11 specimens of the new species and 17 *E. multiplicata* from across the Ouachita Mountains (four of the *E. multiplicata Rag1* sequences were from Lake Catherine State Park).

Additional *E. multiplicata* and outgroup sequences were taken from Genbank (Table 1). Outgroups included other representatives from the "Interior Highland clade" (*E. tynerensis* and *E. spelaea*), all other major lineages of *Eurycea* (*E. bislineata*, *E. longicauda*, *E. neotenes*, and *E. quadridigitata*), and a distant spelerpine outgroup (*Pseudotriton ruber;* Bonett & Chippindale 2004; Bonett *et al.* 2014). Sequences were edited and aligned using Sequencher v4.8 (Gene Codes, Ann Arbor, MI). Both genes are protein coding and contained no stop codons or insertions/deletions. We trimmed the alignments to 880bp for *Cytb* and 1012bp for *Rag1*. The sequences for all taxa were complete, with no missing data.

The most appropriate model of nucleotide substitution for each codon position for each gene was chosen using jModelTest (Guindon & Gascuel 2003; Darriba *et al.* 2012; Table 3). Bayesian phylogenetic analyses were then performed using MrBayes v3.2 (Ronquist & Huelsenbeck 2003). We ran two replicate searches using four chains

each (three hot, one cold) for 15 million generations, sampling every 1000 generations, and assessed support with Bayesian posterior probabilities (BAPP). We assessed stationarity using Tracer v1.5 (Rambaut & Drummond 2007), and the first 3750 trees (25% of generations) were discarded as burn-in; well beyond stationarity. Uncorrected pairwise sequence divergences for *Cytb* and *Rag1* were determined using Mega v5.1 (Tamura *et al.* 2011).

**TABLE 1.** Voucher specimens, localities, and GenBank numbers for Bayesian analysis. Localities are listed as locality number: state abbreviation, county, other descriptive locality information. CG=Campground, Crk=Creek, E=East, N=North, S=South, SP=State Park, W=West, WMA=Wildlife Management Area.

| Species | | Locality #: Location | *Cytb* | *Rag1* |
|---|---|---|---|---|
| *Eurycea multiplicata* | MAS 0187 | 1: AR, Clark, AR, N of Caddo Valley | KJ372351 | --- |
| *Eurycea multiplicata* | RMB 2153 | 2: AR, Conway, AR, Rose Crk | KJ372393 | KJ372328 |
| *Eurycea multiplicata* | MAS 0226 | 3: AR, Faulkner, AR, Camp Robinson | KJ372352 | KJ372319 |
| *Eurycea multiplicata* | RMB 2607 | 4: AR, Garland, AR, Mazarn Crk | KJ372389 | --- |
| *Eurycea multiplicata* | MAS 0007 | 5: AR, Hot Spring, Lake Catherine SP | KJ372344 | KJ372309 |
| *Eurycea subfluvicola* | MAS 0232 | 5: AR, Hot Spring, Lake Catherine SP | KJ372376 | KJ372314 |
| *Eurycea multiplicata* | MAS 0233 | 5: AR, Hot Spring, Lake Catherine SP | KJ372353 | KJ372308 |
| *Eurycea multiplicata* | MAS 0234 | 5: AR, Hot Spring, Lake Catherine SP | KJ372354 | --- |
| *Eurycea multiplicata* | MAS 0447 | 5: AR, Hot Spring, Lake Catherine SP | KJ372359 | --- |
| *Eurycea multiplicata* | MAS 0448 | 5: AR, Hot Spring, Lake Catherine SP | KJ372360 | --- |
| *Eurycea multiplicata* | MAS 0449 | 5: AR, Hot Spring, Lake Catherine SP | KJ372361 | --- |
| *Eurycea multiplicata* | MAS 0450 | 5: AR, Hot Spring, Lake Catherine SP | KJ372362 | --- |
| *Eurycea multiplicata* | MAS 0451 | 5: AR, Hot Spring, Lake Catherine SP | KJ372363 | --- |
| *Eurycea multiplicata* | MAS 0452 | 5: AR, Hot Spring, Lake Catherine SP | KJ372364 | --- |
| *Eurycea multiplicata* | MAS 0819 | 5: AR, Hot Spring, Lake Catherine SP | KJ372366 | KJ372317 |
| *Eurycea multiplicata* | MAS 0820 | 5: AR, Hot Spring, Lake Catherine SP | KJ372367 | --- |
| *Eurycea multiplicata* | MAS 0821 | 5: AR, Hot Spring, Lake Catherine SP | KJ372368 | --- |
| *Eurycea subfluvicola* | MAS 0823 | 5: AR, Hot Spring, Lake Catherine SP | KJ372369 | --- |
| *Eurycea multiplicata* | MAS 0824 | 5: AR, Hot Spring, Lake Catherine SP | KJ372370 | KJ372324 |
| *Eurycea multiplicata* | MAS 0825 | 5: AR, Hot Spring, Lake Catherine SP | KJ372371 | KJ372332 |
| *Eurycea subfluvicola* | MAS 0826 | 5: AR, Hot Spring, Lake Catherine SP | KJ372372 | KJ372331 |
| *Eurycea subfluvicola* | MAS 0827 | 5: AR, Hot Spring, Lake Catherine SP | KJ372373 | --- |
| *Eurycea subfluvicola* | MAS 0828 | 5: AR, Hot Spring, Lake Catherine SP | KJ372374 | KJ372313 |
| *Eurycea subfluvicola* | MAS 0829 | 5: AR, Hot Spring, Lake Catherine SP | KJ372375 | KJ372333 |
| *Eurycea subfluvicola* | MAS 0830 | 5: AR, Hot Spring, Lake Catherine SP | KJ372377 | KJ372334 |
| *Eurycea subfluvicola* | MAS 0831 | 5: AR, Hot Spring, Lake Catherine SP | KJ372378 | --- |
| *Eurycea subfluvicola* | MAS 0832 | 5: AR, Hot Spring, Lake Catherine SP | KJ372379 | KJ372330 |
| *Eurycea subfluvicola* | MAS 0834 | 5: AR, Hot Spring, Lake Catherine SP | KJ372388 | KJ372335 |
| *Eurycea subfluvicola* | MAS 0835 | 5: AR, Hot Spring, Lake Catherine SP | KJ372380 | --- |
| *Eurycea subfluvicola* | MAS 0837 | 5: AR, Hot Spring, Lake Catherine SP | KJ372381 | KJ372312 |
| *Eurycea subfluvicola* | MAS 0838 | 5: AR, Hot Spring, Lake Catherine SP | KJ372382 | KJ372322 |
| *Eurycea subfluvicola* | MAS 0839 | 5: AR, Hot Spring, Lake Catherine SP | KJ372383 | --- |
| *Eurycea subfluvicola* | MAS 0841 | 5: AR, Hot Spring, Lake Catherine SP | KJ372384 | --- |
| *Eurycea subfluvicola* | MAS 0845 | 5: AR, Hot Spring, Lake Catherine SP | KJ372385 | --- |
| *Eurycea subfluvicola* | MAS 0846 | 5: AR, Hot Spring, Lake Catherine SP | KJ372386 | --- |
| *Eurycea subfluvicola* | MAS 0847 | 5: AR, Hot Spring, Lake Catherine SP | KJ372387 | KJ372321 |
| *Eurycea multiplicata* | MAS 0003 | 6 : AR, Logan, Shoal Bay CG | KJ372336 | KJ372311 |
| *Eurycea multiplicata* | MAS 0005 | 7 : AR, Logan, S of Golden City | KJ372337 | KJ372310 |
| *Eurycea multiplicata* | MAS 0420 | 8 : AR, Montgomery, W of Washita | KJ372358 | --- |

*.....continued on the next page*

**TABLE 1.** (Continued)

| Species | | Locality #: Location | *Cytb* | *Rag1* |
|---|---|---|---|---|
| *Eurycea multiplicata* | RMB 2613 | 9 : AR, Montgomery, Long Crk | KJ372394 | KJ372326 |
| *Eurycea multiplicata* | MAS 0176 | 10 : AR, Perry, E of Steve | KJ372350 | KJ372318 |
| *Eurycea multiplicata* | MAS 0023 | 11 : AR, Perry, Garland county border | KJ372346 | --- |
| *Eurycea multiplicata* | UTA_56355 | 12 : AR, Polk, Bogg Springs | AY528334 | --- |
| *Eurycea multiplicata* | MAS 0067 | 13 : AR, Polk, NW of Wolf Pen Gap | KJ372349 | --- |
| *Eurycea multiplicata* | MAS 0252 | 14 : AR, Perry, S of Williams Junction | KJ372356 | --- |
| *Eurycea multiplicata* | RMB 3013 | 15 : AR, Pulaski, Little Rock | KJ372392 | --- |
| *Eurycea multiplicata* | MAS 0016 | 16 : AR, Scott, SW of Beauchamp | KJ372338 | KJ372315 |
| *Eurycea multiplicata* | MAS 0017 | 17 : AR, Scott, Turner Crk | KJ372339 | KJ372320 |
| *Eurycea multiplicata* | MAS 0414 | 18 : AR, Sebastian | KJ372357 | --- |
| *Eurycea multiplicata* | MAS 0019 | 19: AR, Yell, W of Waltreak | KJ372340 | KJ372323 |
| *Eurycea multiplicata* | MAS 0090 | 20: AR, Yell, NW of Waltreak | KJ372343 | KJ372316 |
| *Eurycea multiplicata* | MAS 0020 | 21: AR, Yell, SE of Danville | KJ372345 | --- |
| *Eurycea multiplicata* | UTA_56369 | 22: OK, Atoka, Atoka WMA | AY528332 | --- |
| *Eurycea multiplicata* | MAS 0086 | 23: OK, Choctow, E of Sobel | KJ372342 | KJ372327 |
| *Eurycea multiplicata* | MAS 0057 | 24: OK, Leflore, NW of Zoe | KJ372348 | --- |
| *Eurycea multiplicata* | RMB 2866 | 25: OK, Lelfore, Gap Crk | KJ372390 | --- |
| *Eurycea multiplicata* | RMB 2867 | 26: OK, Leflore, Morris Crk | KJ372391 | --- |
| *Eurycea multiplicata* | MAS 0239 | 27: OK, McCurtain, Beavers Bend SP | KJ372355 | --- |
| *Eurycea multiplicata* | MAS 0463 | 28: OK, McCurtain, N Broken Bow Lake | KJ372365 | --- |
| *Eurycea multiplicata* | MAS 0037 | 29: OK, Pushmataha, Jack fork Crk | KJ372341 | KJ372329 |
| *Eurycea multiplicata* | MAS 0044 | 30: OK, Pushmataha, Honobia Crk WMA | KJ372347 | KJ372325 |
| **Outgroups** | | | | |
| *Eurycea bislineata* | UTA_56411 | NY | AY528402 | EU275784 |
| *Eurycea longicauda* | CM_147803 | PA | AY528403 | AY650121 |
| *Eurycea neotones* | TNHC_60313 | TX | AY528400 | AY650122 |
| *Eurycea spelaea* | UTA_56362 | AR, Boone, Alcohol Springs | AY528390 | KF562666 |
| *Eurycea spelaea* | UTA_56364 | MO, Barry, Rockhouse Cave | AY528393 | --- |
| *Eurycea spelaea* | UTA_56365 | MO, Pulaski, Piquet Cave | AY528395 | --- |
| *Eurycea tynerensis* | UTA_56372 | AR, Conway, Cypress Creek | AY528345 | --- |
| *Eurycea tynerensis* | UTA_56399 | OK, Sequoyah, Near Cookson | AY528367 | --- |
| *Eurycea tynerensis* | UTA_56404 | OK, Sequoyah, Little Lee Crk | AY528387 | --- |
| *Eurycea tynerensis* | UTA_53860 | OK, Cherokee, Camp Egan | --- | KF562675 |
| *Eurycea quadradigita* | UTA_56412 | TX | AY528401 | --- |
| *Pseudotriton ruber* | ASB 130 | NY: Sullivan, Adams Road | AY528404 | --- |
| *Pseudotriton ruber* | RMB 3705 | NC, Graham, Rattler Ford Campground | --- | KF562700 |

To estimate relative divergence dates, we used the spelerpine dataset of Bonett *et al.* (2014), which used two unlinked genes (mtDNA and *Rag1*) for 75 taxa; including outgroups. To avoid missing data between taxa, we sequenced a portion of the mitochondrial gene NADH dehydrogenase subunit 4 (*Nd4*) for one specimen of the new species, and trimmed each gene alignment (*Cytb*=589, *Nd4*=630, and *Rag1*=1012; Table 2). The best-fitting models of nucleotide substitution were determined using jModelTest (Table 3). We followed the methods of Bonett *et al.* (2014) and the *BEAST function (Heled & Drummond 2010) in BEAST v1.7.5 (Drummond *et al.* 2012). *BEAST analyses were run three times independently for 20 million generations. Stationarity of likelihood values was evaluated using Tracer v1.5 (Rambaut & Drummond 2007) and the first 25% of generations were discarded from each run and remaining generations were combined using LogCombiner v1.7.5.

**TABLE 2.** Voucher specimens, localities, and GenBank numbers. Localities are listed as state abbreviation: county, other descriptive locality information. Cv.= Cave, Crk.=Creek, R.= River, Sprg.=Spring, SF=State Forest, SP=State Park, NF=National Forest.

| **Species** | | **Location** | ***Cytb*** | ***Nd4*** | ***Rag1*** |
|---|---|---|---|---|---|
| Spelerpini | | | | | |
| *Eurycea aquatica* | RMB3702 | GA: Chattooga, Moseley Sprg. | KF562543 | KF562594 | KF562645 |
| *Eurycea bislineata* | UTA-A56411 | NY: Chenango, Genegantslet R. | AY528402 | AY528327 | AY691606 |
| *Eurycea chamberlaini* | TBC | NC: Pitt, Greenville, Elm Street Park | KF562544 | KF562595 | KF562646 |
| *Eurycea chisholmensis* | PTC/DMH90-3 | TX: Bell, Salado (main) Sprgs. | KF562545 | KF562596 | KF562647 |
| *Eurycea cirrigera* 1 | UTA-A56672 | FL | KF562546 | KF562597 | KF562648 |
| *Eurycea cirrigera* 2 | MAS0318 | GA: Polk | KF562547 | KF562598 | KF562649 |
| *Eurycea cirrigera* 3 | MAS0281 | TN: Cannon, Woodbury | KF562548 | KF562599 | KF562650 |
| *Eurycea guttolineata* | RMB2493 | SC: Berkeley, Francis Marion NF | KF562549 | KF562600 | KF562651 |
| *Eurycea junaluska* | UTA-A53897 | TN: Sevier, Little R. | KF562550 | KF562601 | FJ750246 |
| *Eurycea latitans* | AGG1798 | TX: Kendall, Cascade Caverns | KF562551 | KF562602 | KF562652 |
| *Eurycea l. longicauda* | CM147803 | PA: Washington | AY650121 | AY528328 | AY528403 |
| *Eurycea l. melanopleura* | RMB2840 | AR: Lawrence, Bubbling Sprg. | KF562552 | KF562603 | KF562653 |
| *Eurycea lucifuga* | UTA-A53867 | AR: Independence, Cushman Cv. | KF562553 | KF562604 | KF562654 |
| *Eurycea multiplicata* 1 | UTA-A56353 | AR: Saline, Williams Crk. | AY528339 | AY528264 | AY691707 |
| *Eurycea multiplicata* 2 | UTA-A56367 | OK: Choctaw, near Ft. Towson | AY528330 | AY528255 | KF562655 |
| *Eurycea nana* | PTC97-2 | TX: Hays: San Marcos Sprg. | KF562554 | KF562605 | KF562656 |
| *Eurycea naufragia* | PTC09-1 | TX: Williamson, Swinbank Sprg. | KF562555 | KF562606 | KF562657 |
| *Eurycea neotenes* | TNHC60313 | TX: Bexar, Helotes Sprg. | AY528400 | AY528325 | AY650122 |
| *Eurycea pteropila* | AGG1858 | TX: Hays, Sycamore Sprg. | KF562556 | KF562607 | KF562658 |
| *Eurycea quadridigitata* 1 | PTC96-16 | AL | KF562557 | KF562608 | KF562659 |
| *Eurycea quadridigitata* 2 | RMB3665 | AR: Calhoun, Hurricane Crk. | KF562558 | KF562609 | KF562660 |
| *Eurycea quadridigitata* 3 | RMB3117 | LA: Washington, Stubbs Crk. | KF562559 | KF562610 | KF562661 |
| *Eurycea quadridigitata* 4 | PTC95-24 | NC | KF562560 | KF562611 | KF562662 |
| *Eurycea quadridigitata* 5 | UTA-A56412 | TX: Tyler | AY528401 | AY528326 | AY691708 |
| *Eurycea rathbuni* | PTC91-13 | TX: Hays, Rattlesnake Cv.. | KF562561 | KF562612 | KF562663 |
| *Eurycea sosorum* | E31 | TX: Travis: Barton Sprg., Eliza outlet | KF562562 | KF562613 | KF562664 |
| *Eurycea* sp. Devil's River | AGG1989 | TX: Val Verde, Finnegan Sprg. | KF562563 | KF562614 | KF562665 |
| *Eurycea spelaea* 1 | UTA-A56362 | AR: Boone, Alcohol Sprg. | AY528390 | AY528315 | KF562666 |
| *Eurycea spelaea* 2 | UTA-A53846 | OK: Mayes, Pipe Sprg. | AY528397 | AY528322 | KF562667 |
| *Eurycea subfluvicola* | MAS 0232 | AR: Hot Spring, Lake Catherine SP | KJ372376 | KJ372307 | KJ372314 |
| *Eurycea tonkawae* | DMH88-144 | TX: Travis, Stillhouse Hollow Sprg. | KF562564 | KF562615 | KF562668 |
| *Eurycea tridentifera* | TNHC-54574 | TX: Comal, Honey Creek Cv. | KF562565 | KF562616 | KF562669 |
| *Eurycea troglodytes* 1 | PTC90-90 | TX: Bandera, Sutherland Hollow | KF562567 | KF562618 | KF562671 |
| *Eurycea troglodytes* 2 | PTC89-67 | TX: Bandera, Sabinal River Sprg. | KF562566 | KF562617 | KF562670 |
| *Eurycea troglodytes* 3 | AHP3051 | TX: Kerr, Fessenden Sprg. | KF562568 | KF562619 | KF562672 |
| *Eurycea tynerensis* 1 | UTA-A56375 | AR: White, Little Crk. | AY528348 | AY528273 | KF562673 |

……*continued on the next page*

**TABLE 2.** (Continued)

| Species | | Location | *Cytb* | *Nd4* | *Rag1* |
|---|---|---|---|---|---|
| *Eurycea tynerensis* 2 | RMB2775 | MO: Christian, Buseik SF | AY528385 | AY528310 | KF562674 |
| *Eurycea tynerensis* 3 | UTA-A53860 | OK: Cherokee, Rock Crk. | AY528374 | AY528299 | KF562675 |
| *Eurycea tynerensis* 4 | UTA-A56399 | OK: Cherokee, Cookson | AY528367 | AY528292 | KF562676 |
| *Eurycea tynerensis* 5 | UTA-A56402 | OK: Sequoyah, Tin Cup Crk. | AY528368 | AY528293 | KF562677 |
| *Eurycea tynerensis* 6 | UTA-A56404 | OK: Sequoyah, Little Lee Crk. | AY528387 | AY528312 | KF562678 |
| *Eurycea wallacei* | TNHC-53821 | FL: Jackson | KF562583 | KF562634 | KF562693 |
| *Eurycea waterlooensis* | AGG1995 | TX: Travis: Barton Sprg. | KF562569 | KF562620 | KF562679 |
| *Eurycea wilderae* | MAS0316 | GA: Union, Wolf Crk. | KF562570 | KF562621 | KF562680 |
| *Gyrinophilus gulolineatus* | CR1 | TN: Knox, Christian Cv. | KF562571 | KF562622 | KF562681 |
| *Gyrinophilus p. palleucus* | CH3 | TN: Franklin, Custard Hollow Cv. | KF562574 | KF562625 | KF562684 |
| *Gyrinophilus p. necturoides* 1 | B52 | TN: Coffee, Blowing Spr Cv. | KF562572 | KF562623 | KF562682 |
| *Gyrinophilus p. necturoides* 2 | L2 | TN: Coffee, Lust Cv. | KF562573 | KF562624 | KF562683 |
| *Gyrinophilus p. danielsi* 1 | MAS0319 | NC: Yancey, Black Mt. Campground | KF562576 | KF562627 | KF562686 |
| *Gyrinophilus p. danielsi* 2 | ASB0030 | NC: Wilkes | KF562577 | KF562628 | KF562687 |
| *Gyrinophilus p. dunni* | MAS0217 | SC: Aconi, Winding Stairs Road | KF562578 | KF562629 | KF562688 |
| *Gyrinophilus p. porphyriticus* 1 | ASB0021 | AL | KF562575 | KF562626 | KF562685 |
| *Gyrinophilus p. porphyriticus* 2 | C4 | TN: Knox, Cruze Cv. | KF562579 | KF562630 | KF562689 |
| *Gyrinophilus p. porphyriticus* 3 | MF2 | TN: Knox, Mudflats Cv. | KF562580 | KF562631 | KF562690 |
| *Gyrinophilus p. porphyriticus* 4 | P2 | TN: DeKalb Co., Pauley Cv. | KF562581 | KF562632 | KF562691 |
| *Gyrinophilus subterraneus* | MLN0551 | WV: Greenbrier, General Davis Cv. | KF562582 | KF562633 | KF562692 |
| *Pseudotriton m. diasticus* | H91-26 | KY: Lincoln | KF562584 | KF562635 | KF562694 |
| *Pseudotriton m. flavissimus* | H86-30 | AL: Macon, Tuskegee NF | KF562585 | KF562636 | KF562695 |
| *Pseudotriton m. montanus* | MVZ-137301 | NC: Wake, Falls of the Neuse | KF562586 | KF562637 | KF562696 |
| *Pseudotriton r. nitidus 1* | MVZ-143976 | GA: Emmanuel | KF562587 | KF562638 | KF562697 |
| *Pseudotriton r. nitidus 2* | ASB0031 | NC: Watauga | KF562588 | KF562639 | KF562698 |
| *Pseudotriton r. schencki* | RMB3705 | NC: Graham, Rattler Ford Campground | KF562590 | KF562641 | KF562700 |
| *Pseudotriton r. ruber 1* | ASB0130 | NY: Sullivan, Adams Road | AY528404 | AY528329 | AY650123 |
| *Pseudotriton r. ruber 2* | MAS0320 | TN: Cumberland, Staples Sprg. Brch. | KF562589 | KF562640 | KF562699 |
| *Pseudotriton r. vioscai 1* | ASB0088 | AL | KF562591 | KF562642 | KF562701 |
| *Pseudotriton r. vioscai 2* | LSUMZ-2182 | MS: Clay, Shinburne Crk. | KF562592 | KF562643 | KF562702 |
| *Stereochilus marginatus* | ASB072 | SC: Charleston, Santee Coastal Res. | AY691753 | AY691797 | AY691713 |
| *Ursplerpes brucei* | RMB3708 | GA: Stephens, Panther Crk. | KF562593 | KF562644 | KF562703 |
| **Outgroups** | | | | | |
| *Aneides aeneus* | JC32 | WV: Kanawha, Kanawha SF | AY691742 | AY691786 | AY691701 |
| *Bolitoglossa helmrichi* | UTA-A51457 | Guatemala | AY691755 | AY691799 | AY650124 |
| *Desmognathus brimleyorum* | UTA-A53882 | AR: Garland, Cearly Crk. | AY691737 | AY691781 | AY691697 |
| *Ensatina eschscholtzii* | UTA-A56606 | OR: Curry | AY691743 | AY691787 | AY691702 |
| *Hemidactylium scutatum* | UTA-A56604 | AR: Saline, Williams Crk. | AY691751 | AY691795 | AY691711 |
| *Plethodon cinereus* | ASUMZ23844 | NC: Caldwell, Dixon Crk. | AY691745 | AY691789 | AY691703 |
| *Plethodon vehiculum* | UTA-A56610 | WA: Lewis, Trib. of Tilton R. | AY691760 | AY691804 | AY691716 |
| *Amphiuma means* | ASUMZ23768 | FL: Levy, Gulf Hammock | AY691722 | AY691766 | AY650127 |

**TABLE 3.** Models applied to each data partition for Bayesian phylogenetic analyses and divergence time estimation.

| Gene/partition-MrBayes-Bayesian Inference | # of positions in partition | Model | rates |
|---|---|---|---|
| *Cytb*: alignment pos 1, codon pos 2 | 294 | HKY | propinv |
| *Cytb*: alignment pos 2, codon pos 3 | 293 | GTR | gamma |
| *Cytb*: alignment pos 3, codon pos 1 | 293 | GTR | invgamma |
| *Rag1*: alignment pos 1, codon pos 1 | 338 | HKY | propinv |
| *Rag1*: alignment pos 2, codon pos 3 | 337 | HKY | gamma |
| *Rag1*: alignment pos 3, codon pos 2 | 337 | HKY | propinv |
| Gene/partition-*BEAST-Divergence chronogram | | | |
| *Cytb*: alignment pos 1, codon pos 2 | 197 | HKY | gamma |
| *Cytb*: alignment pos 2, codon pos 3 | 196 | GTR | gamma |
| *Cytb*: alignment pos 3, codon pos 1 | 196 | SYM | gamma |
| *Nd4*: alignment pos 1, codon pos 1 | 210 | HKY | invgamma |
| *Nd4*: alignment pos 2, codon pos 2 | 210 | GTR | invgamma |
| *Nd4*: alignment pos 3, codon pos 3 | 210 | GTR | gamma |
| *Rag1*: alignment pos 1, codon pos 1 | 338 | GTR | gamma |
| *Rag1*: alignment pos 2, codon pos 3 | 337 | HKY | propinv |
| *Rag1*: alignment pos 3, codon pos 2 | 337 | GTR | gamma |

Fragment lengths for MrBayes analyses: *Cytb* = 880bp, *Rag1* = 1012bp
Fragment lengths for *BEAST analysis: *Cytb* = 589bp, *Nd4* = 630bp, *Rag1* = 1012bp; Total = 2,231

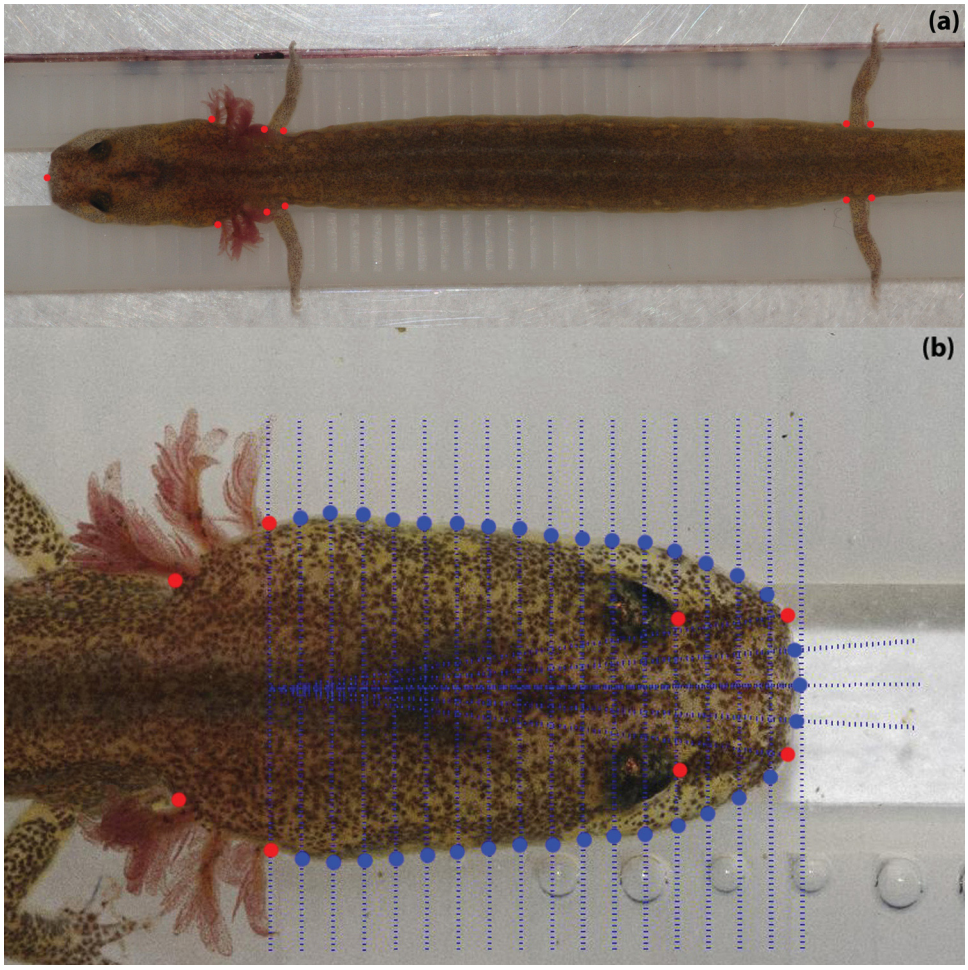


**FIGURE 2**. Position of landmarks (red) and semilandmarks (blue) used in Principle Component Analysis (PCA) and the Goodall's F test for body (a) and head (b) data. The fan and comb feature in MakeFan7 are also shown to demonstrate how semilandmarks were selected.

## Results

***Eurycea subfluvicola* sp. nov.**
Ouachita Streambed Salamander

**Holotype**. The holotype is an adult female collected by MAS and KJI on 25-March 2013 and deposited at the Museum of Vertebrate Zoology, University of California, Berkeley (MVZ 269485; Fig. 3). Detailed measurements (mm) post preservation is listed in Table 4. Though slightly faded in preservation, the holotype is similar in coloration and pattern as described in the species diagnosis. The holotype contains nine enlarged oviductal eggs that are visible through the venter.

**TABLE 4**. Detailed measurements (mm) of *Eurycea subfluvicola* holotype.

| | |
|---|---|
| Total Length | 67.9 |
| Standard Length (SVL) | 42.0 |
| Head length (snout to gular fold) | 5.1 |
| Head depth at posterior angle of jaw | 1.8 |
| Head width | 5.0 |
| Eye to nostril (left) | 1.5 |
| Eye diameter (horizontal) | 0.8 |
| Interorbital distance | 1.4 |
| Snout to forelimb insertion | 9.5 |
| Trunk Length | 27.5 |
| Width at mid-body | 5.5 |
| Depth at mid-body | 4.2 |
| Tail length | 26.0 |
| Tail width at base | 3.4 |
| Tail depth at base | 3.0 |
| Forelimb length to tip of longest digit (left) | 4.6 |
| Hindlimb length to tip of longest digit (left) | 5.4 |

**Paratypes.** Paratypes are one formalin fixed specimen (AMNH A191438) and seven cleared and stained specimens deposited at the American Museum of Natural History in New York City, New York (AMNH A191439-A191441) and the Museum of Vertebrate Zoology (MVZ 269485-269489).

**Type locality.** A unnamed first order tributary of Slunger Creek, located in the Trap Mountains, a subdivision in the southeastern portion of the Ouachita Mountain physiographic province, Hot Spring County, Arkansas, USA. This locality is within the confines of Lake Catherine State Park.

**Etymology.** The specific name, s*ubfluvicola,* is an adjective derived from the Latin prefix, *sub*- meaning "below", *fluvius* which is "a stream", and *colo* meaning "to dwell." The translation then is "dwells below the stream", in reference to its existence below the streambed during xeric conditions.

**Diagnosis.** This species is assigned to the genus *Eurycea* based on strong molecular phylogenetic evidence, and overall similarity to other larval *Eurycea*. The genus *Eurycea* is highly heteromorphic, with no single character to diagnosis it from other spelerpine genera (Wake 1966; Camp et al. 2009; Adams et al. 2009). However, members are consistently monophyletic in molecular phylogenetic studies (Camp *et al*. 2009; Bonett *et al.* 2014).

The dorsum of *Eurycea subfluvicola* is primarily uniform amber/yellow background color, pigmented with numerous dark brown melanophores, which create irregularly shaped blotches throughout the dorsum and flanks (Fig. 3). In most individuals irregularly spaced spots are formed by the absence of melanophores along the dorso-lateral region of the trunk, possibly indicative of the lateral line. The semi-transparent venter is unpigmented, except for a few widely dispersed melanophores beneath the tail. Dorsal and ventral coloration is separated by a sharply defined ventral-lateral boundary along the trunk.

**FIGURE 3.** Dorsal and ventral images of the female holotype (MVZ 269485) of *Eurycea subfluvicola.* Preserved October 2013.

Morphological characteristics that distinguish *Eurycea subfluvicola* from syntopic *E. multiplicata* larvae are: 1) *Eurycea subfluvicola* is paedomorphic (i.e. presence of mature testes and ova, and deposition of fertilized eggs while in the larval form) and, based on our samples, is sexually mature by at least 32mm SVL. *Eurycea multiplicata* are never known to be paedomorphic and typically metamorphose between 24 to 41mm SVL. 2) Head and body shape differ significantly between the two species. PCAs show only minimal overlap in morphospace between body shape, and no overlap in head shape (Fig. 4). Additionally, the Goodall's F-test on geometric morphometric data was highly significant for heads, and bodies ($F_{82,2706}$ =35.85, $p<0.001$ and $F_{18,630}$=13.60, $p<0.001$). The same pattern was also seen when comparing only small *E. subfluvicola* (<40mm SVL) to *E. multiplicata* larvae ($F_{82,1558}$=25.53, $p<0.001$, and $F_{18,324}$=4.94, $p<0.001$). 3) Relative to SVL, *E. subfluvicola* has a longer trunk, and a shorter and narrower head compared to *E. multiplicata* larvae (Table 5). This may be driven by the fact that *E. subfluvicola* has a very long trunk relative to its head, but these differences are not significant. 4) In profile, *Eurycea subfluvicola* has a flat head and longer snout (ANCOVA, $p <0.001$; Table 5) with relatively smaller in diameter and depressed eyes, compared to *E. multiplicata* larvae (ANCOVA, $p <0.05$; Fig. 5, Table 5). 5) Individuals of *E.subfluvicola* are more attenuate in body form than *E. multiplicata* larvae, having smaller diameter trunks and tail widths, with the latter being highly significant (ANCOVA, $p < 0.001$; Table 5). 6) *E. multiplicata* have many more iridophores in their irises, whereas *E. subfluvicola* has reduced coloration in its iris making the eye primarily black in color. 7) A prominent black stripe of pigmentation is present along the lateral side of the snout and head in *E. multiplicata*, this stripe is absent in *E. subfluvicola* (Fig. 5).

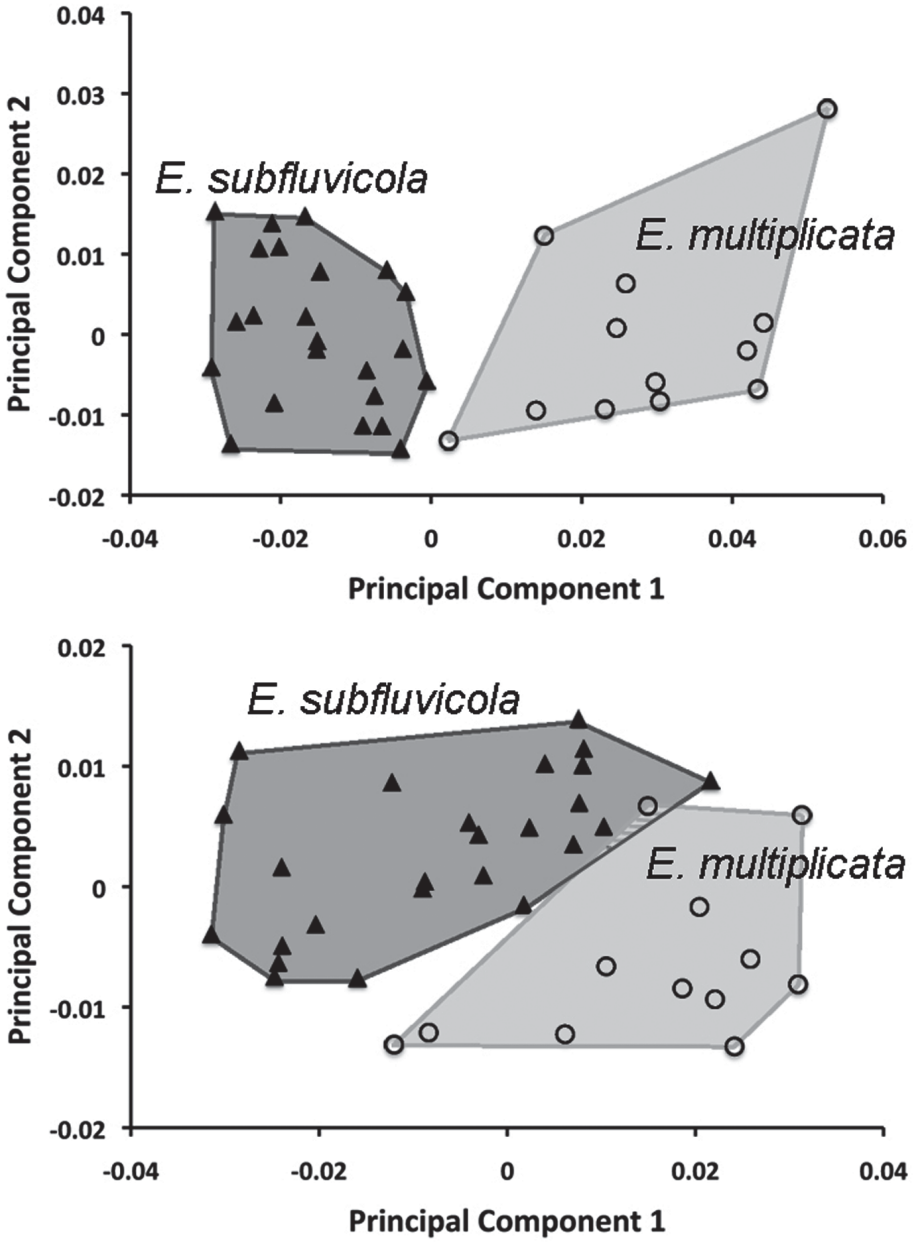


**FIGURE 4.** Principle Component Analysis (PCA) of adult *E. subfluvicola* (dark grey) and larval *E. multiplicata* (light grey), demonstrating minimal overlap in head (top) and body (bottom) morphospace. Landmarks and semilandmarks are shown in Figure 2.

Larval morphology of plethodontids is highly conserved, and *E. subfluvicola* is very similar to the larvae of other species of *Eurycea* (Wake 1966). Trunk vertebral counts (between atlas and sacrum) are 21-22 (n=7, mode=21), whereas syntopic and nearby populations of *E. multiplicata* (n=17) have 21 vertebrae. The trunk vertebrae of *E. subfluvicola* also appears to be longer and narrower than *E. multiplicata* (Fig. 6). The phalanges are well ossified with cartilaginous caps, exhibiting the standard plethodontid phalangeal formulae: 1-2-3-2 for the forelimbs, and 1-2-3-3-2 for the hindlimbs (Shubin & Wake 2003). Carpal counts are eight, and tarsal counts are nine.

The quadrate and squamosal, which are part of the mandibular suspensorium, are collectively more robust in *E. subfluvicola* than in *E. multiplicata* (Fig. 6). As in most spelerpine larval forms, *E. subfluvicola* contain 3 cartilaginous epibranchials, and in some individuals these structures are partially ossified (two of five specimens), whereas no ossification is apparent in *E. multiplicata* larvae (n=10; Fig. 6).

**TABLE 5.** Standard measurements of *E. subfluvicola* and *E. multiplicata*. All standard morphological measurements were made to the nearest 0.1mm. TL=Trunk Length, SVL=Snout vent length, HL=head length, HW=head width, EN=eye to nostril distance, ED=eye diameter, ID=interorbital distance. TRW=trunk width anterior to forlimbs, TW=tail width.

| Measurement Stats | *E. subfluvicola* (n=24)(n=8 pairs for ED) | *E. multiplicata* larvae (n=12)(n=8 pairs for ED) |
|---|---|---|
| Mean TL/SVL ± SD | 0.632 ± 0.019 | 0.601 ± 0.020 |
| Range TL/SVL | 0.581–0.667 | 0.558–0.634 |
| Range | 19.1–31.7 | 10.5–26.0 |
| Mean HL/SVL ± SD | 0.160 ± 0.009 | 0.182 ± 0.014 |
| Range HL/SVL | 0.146–0.186 | 0.159–0.199 |
| Range | 5.6–7.5 | 3.7–6.7 |
| Mean HW/SVL ± SD | 0.128 ± 0.007 | 0.151 ± 0.012 |
| Range HW/SVL | 0.117–0.143 | 0.127–0.175 |
| Range | 4.5–6.1 | 3.2–5.7 |
| Mean TRW/SVL ± SD | 0.090 ± 0.004 | 0.097 ± 0.007 |
| Range TRW/SVL | 0.085–0.095 | 0.088–0.115 |
| Range | 2.9–4.2 | 2.1–3.6 |
| Mean TW/SVL ± SD | 0.081 ± 0.004 | 0.089 ± 0.005 |
| Range TW/SVL | 0.074–0.085 | 0.080–0.097 |
| Range | 2.6–4.0 | 1.6–3.5 |
| Mean EN/SVL ± SD | 0.042 ± 0.002 | 0.039 ± 0.003 |
| Range EN/SVL | 0.037–0.045 | 0.035–0.046 |
| Range | 1.3–2.0 | 0.8–1.5 |
| Mean ED/SVL ± SD | 0.022 ± 0.002 | 0.032 ± 0.004 |
| Range ED/SVL | 0.019–0.025 | 0.027–0.040 |
| Range | 0.7–1.0 | 0.7–1.0 |
| Mean ID/SVL ± SD | 0.035 ± 0.003 | 0.040 ± 0.003 |
| Range ID/SVL | 0.031–0.040 | 0.035–0.046 |
| Range | 1.1–2.0 | 0.7–1.4 |

**Phylogenetic Reconstruction and Divergence Time Estimation.** As in previous phylogenetic analyses our *Cytb* phylogeny strongly supports the monophyly of the *Eurycea multiplicata* complex, which includes *E. multiplicata*, *E. tynerensis*, *E. spelaea* (Bonett & Chippindale 2004; Bonett *et al.* 2014), and now *E. subfluvicola* (Fig. 7a). *Eurycea subfluvicola* is sister to the clade that includes all representative *E. multiplicata* populations from throughout the Ouachita Mountains (BAPP=0.99). Uncorrected pairwise sequence divergence between *E. subfluvicola* and *E. multiplicata* ranges from 13.5-16.6% (mean=15%) for *Cytb*. This is more than two to five times the divergence of many recognized sister species of plethodontid salamanders (Chippindale *et al.* 2000; Hillis *et al.* 2001; Jockusch *et al.* 2002; Garcia-Paris *et al.* 2000; Vences & Wake 2007).

The phylogeny of *Rag1* (Fig. 7b) also shows *E. subfluvicola* as sister to all *E. multiplicata* (BAPP=0.77), with relatively high sequence divergence between these taxa for *Rag1*, uncorrected p=0.9-2.3% (mean=1.4%), which is considerable given that it is a much more conserved nuclear gene (Hoegg *et al.* 2004). What is most informative about the DNA sequence data is that it shows *E. subfluvicola* is highly divergent from syntopic *E. multiplicata* for both a mitochondrial (n=32 specimens) and a nuclear (n=15 specimens) gene (Fig. 7).

Bayesian species-tree and divergence time estimation based on mitochondrial (*Cytb* and *Nd4*) and nuclear (*Rag1*) gene trees shows strong support for the sister relationship of *E. subfluvicola* and *E. multiplicata* (Fig. 8). If we constrain the basal node of all spelerpine plethodontids to be 49 million years old (Bonett *et al.* 2014), then the divergence between *E. subfluvicola* and *E. multiplicata* dates to approximately 18 million years ago.

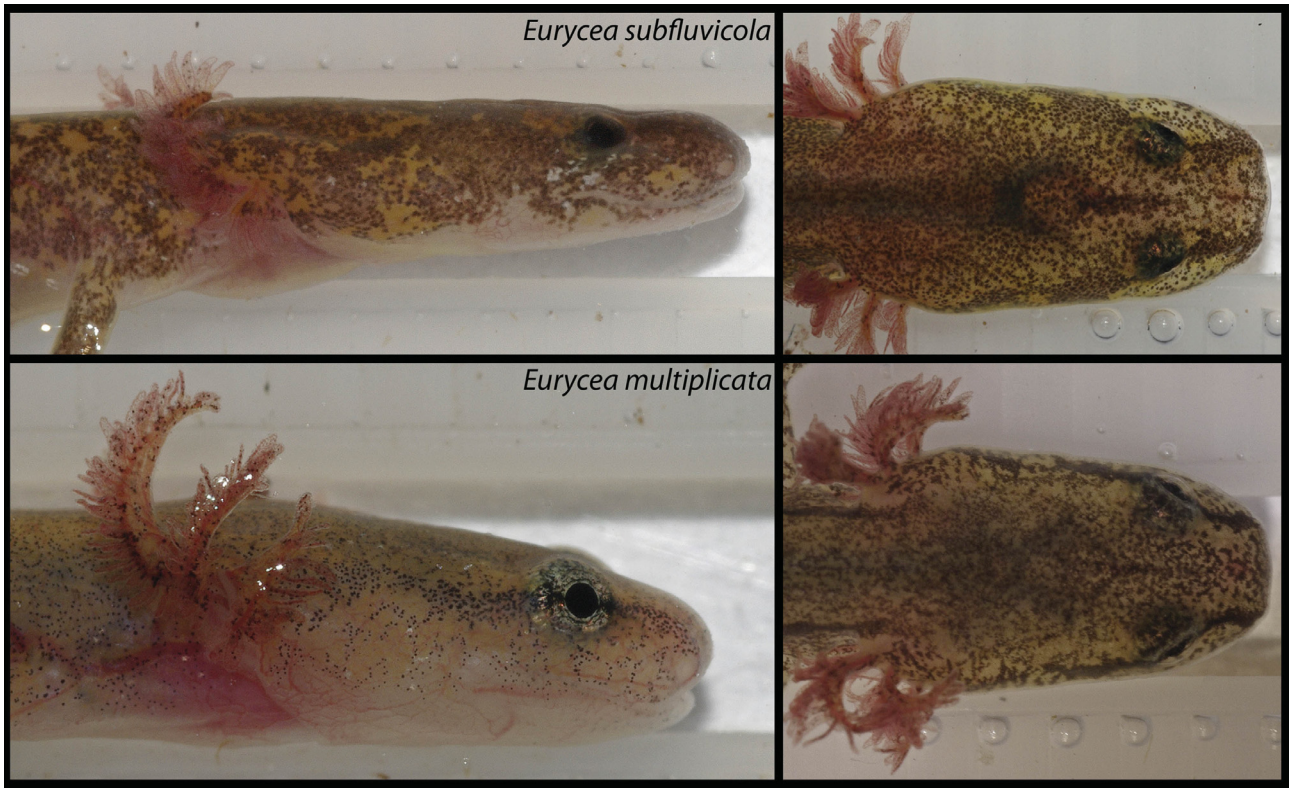


**FIGURE 5**. Side by side lateral and dorsal images of adult *E. subfluvicola* (top) and larval *E. multiplicata* (bottom) illustrating head shape differences between syntopic species.

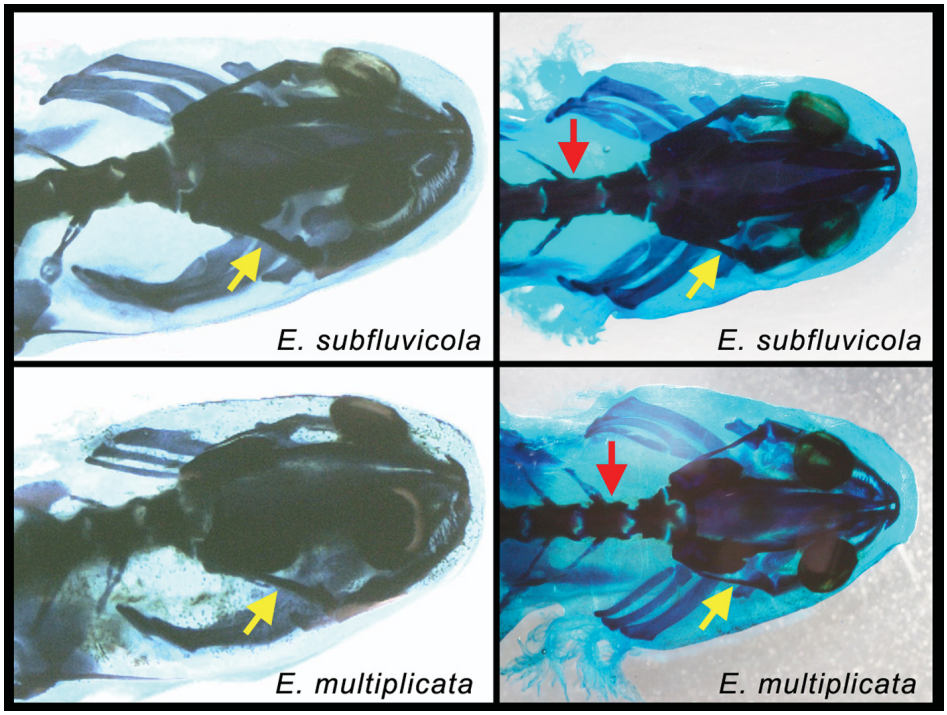


**FIGURE 6**. Cleared and stained images of *E. subfluvicola* (top) and *E. multiplicata* (bottom) skulls. Yellow arrows indicate quadrate and squamosal bones which are collectively more robust in *E. subfluvicola* than *E. multiplicata*. Red arrows point to trunk vertebrae which appear more elongate in *E. subfluvicola* than *E. multiplicata*.

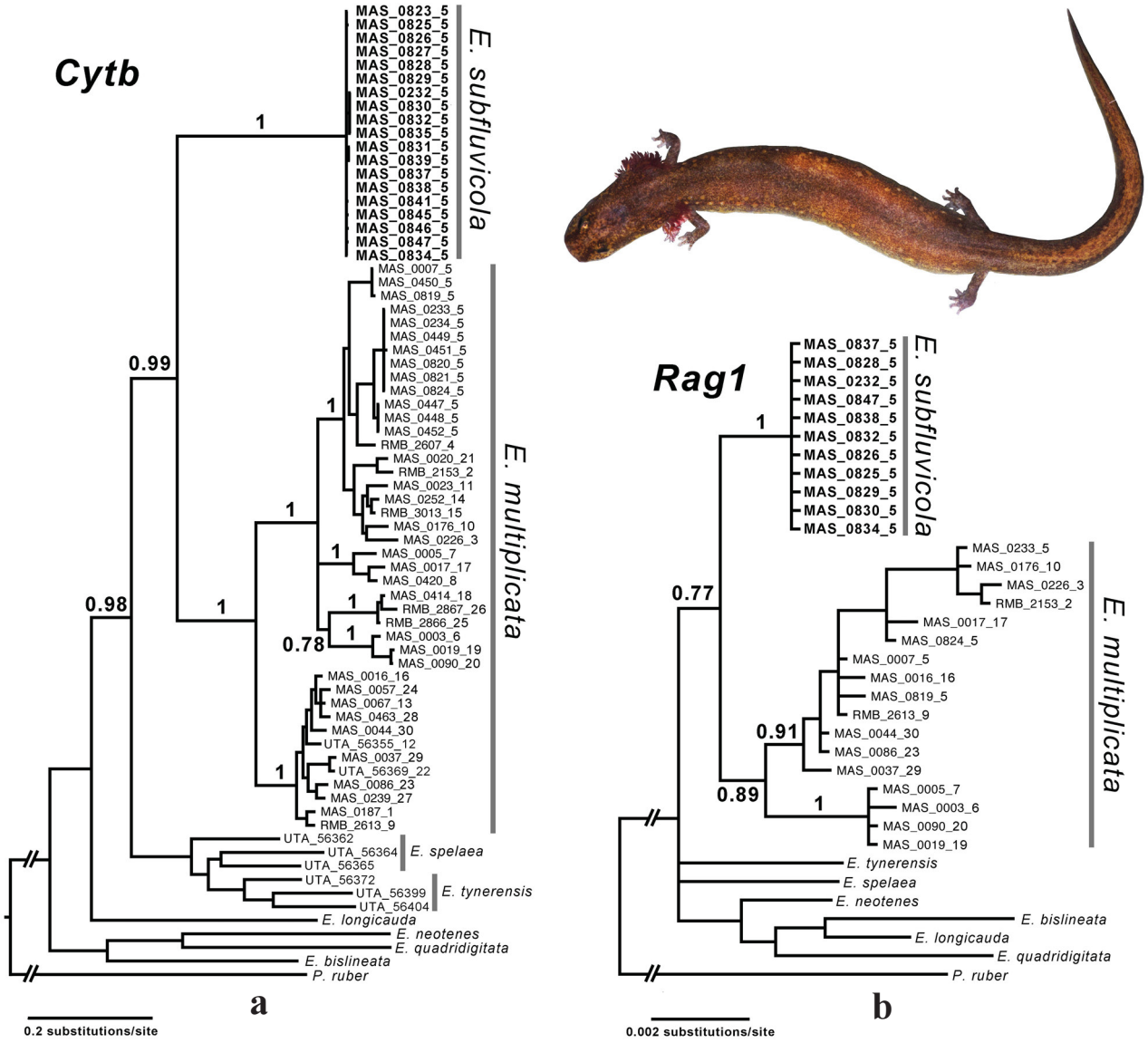


**FIGURE 7.** The 50% majority rule phylogram from a Baysian inference analysis of mitochondrial and nuclear genes. Partitioned Bayesian analyses of *Cytb* (a) and *Rag1* (b), based on 15 million generations, showing *E. subfluvicola* (bold) and its sister taxon *E. multiplicata*. Posterior probabilities indicating node support are shown for major nodes of ingroup clades. For ingroup taxa, the label is the field number followed by the locality number (1 through 30; Figure 1; Table 1). Note that locality number 5 includes *E. subfluvicola* and sympatric *E. multiplicata*. *E=Eurycea* and *P=Pseudotriton*.

**Variation**. Variation is based on 24 specimens of *E. subfluvicola* (8 males, 16 females). All 24 individuals were adults, based on presence of mature or maturing egg follicles, or enlarged testes (Figs. 3 and 9). Male SVL ranged in size from 34.9-45.7mm (mean=40.8) and between 31.4-48.0mm (mean=41.1) for females. There are no somatic differences between the sexes, and only minor differences in coloration among individuals. We found 0.1-0.3% nucleotide divergence (uncorrected p) in *Cytb* and 18 sites with heterozygous positions across all individuals of *E. subfluvicola* for *Rag1*.

**Distribution and Habitat**. The known distribution of *Eurycea subfluvicola* is currently limited to the Trap Mountains, a range of generally east-west trending ridges in the southeastern portion of the Ouachita Mountain physiographic province. These uplands are composed of Paleozoic marine sedimentary rocks; principally the Devonian/Mississippian Arkansas Novaculite and younger Mississippian Stanley Shale formations. Stream valley alluvium deposits overlie the Stanley Shale and predominantly consist of Arkansas Novaculite gravels and cobbles. *Eurycea subfluvicola* is only known from one locality where it can be found in two nearby sites. The two sites are a 15m section of Slunger Creek and a 50m section of an unnamed tributary within the Slunger Creek alluvial valley;

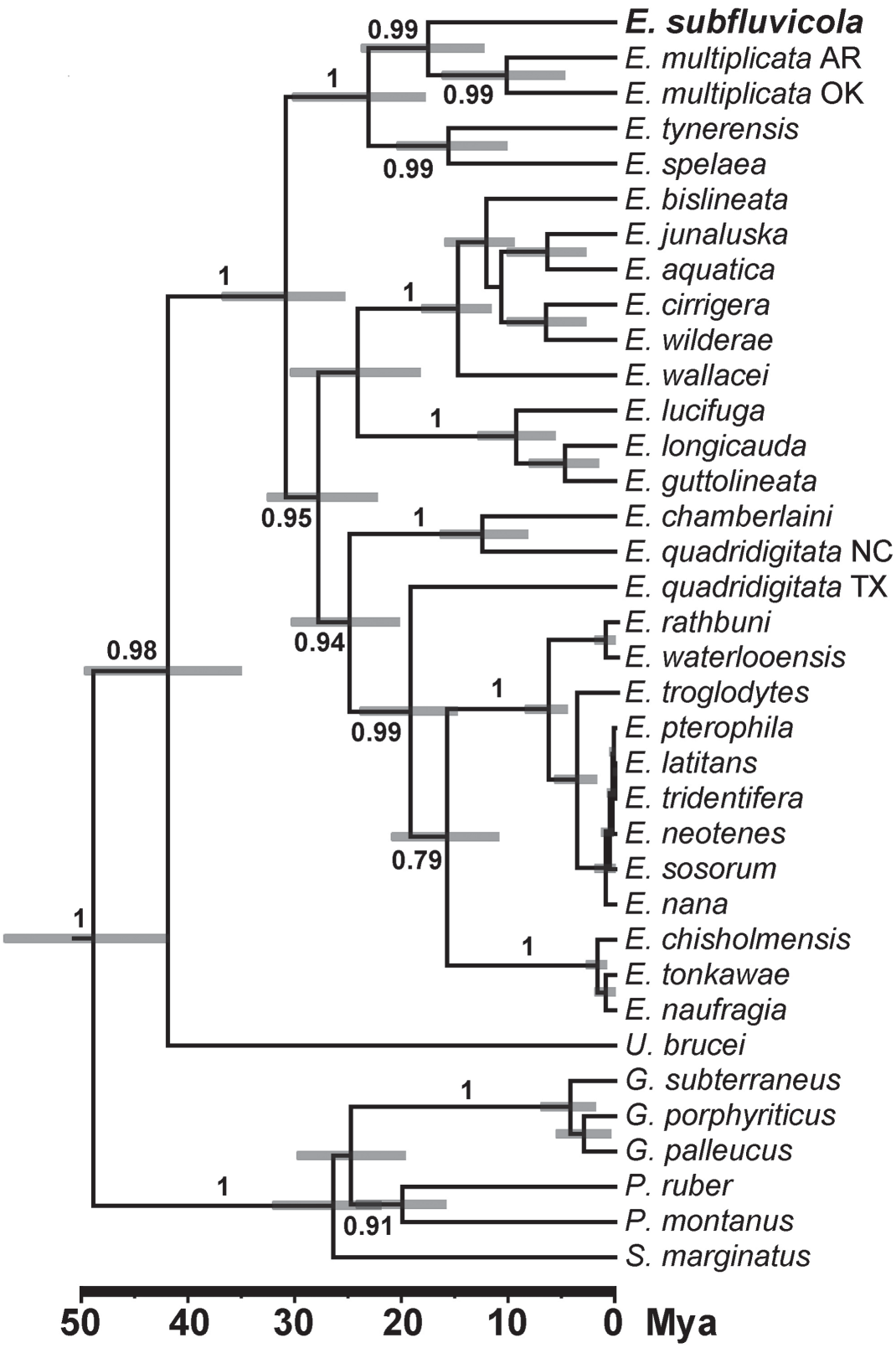


**FIGURE 8.** Bayesian species tree chronogram of spelerpines plethodontid salamanders including *E. subfluvicola* (bold). The species tree and divergence times estimates from *BEAST are based on mitochondrial (*Cytb* and *Nd4*) and nuclear gene (*Rag1*) trees (see Methods). Numbers subtending each node are posterior probabilities of node support for major lineages. Node bars indicate 95% highest posterior density on divergence dates. Some redundant taxa were pruned. *E=Eurycea*, *G=Gyrinophilus*, *P=Pseudotriton, S=Stereochilus,* and *U=Urspelerpes*. AR=Arkansas, NC=North Carolina, OK=Oklahoma, and TX=Texas.

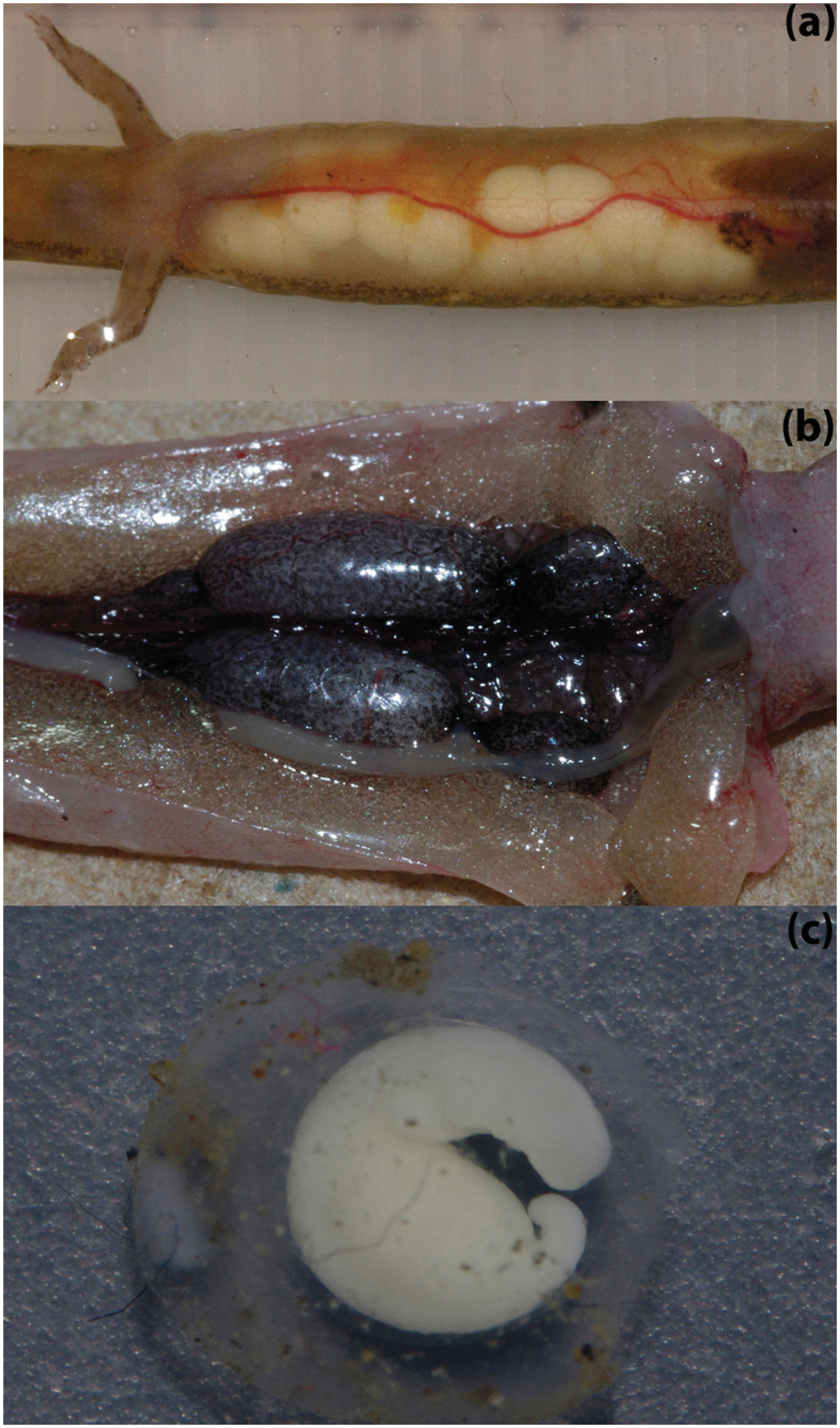


**FIGURE 9**. Images of reproductive status in *E. subfluvicola.* (a) Female with developing ova in ovaries. (b) Male with enlarged testicular lobes, and smaller lobules in development. (c) Developing embryo of *E. subfluvicola* (16 days old).

approximately 135m apart from one another. During our 2013 surveys, we found more individuals of *E. subfluvicola* (approximately 50 observations) in the longer stream section, while fewer individuals were found in the smaller stream section (<10 observations). Surface stream flows are seasonally ephemeral in these two stream reaches. However, surface flow persists, both upstream and downstream, when the streambed is completely dry at these two sites. This suggests a subsurface flow in the stream channels or hyporheic zone connectivity between these points, which may provide habitat for *E. subfluvicola* during periods of low rainfall. Our ability to observe *E. subfluvicola* is dependent upon the rise of the water table during winter and spring, which is similar to the seasonal observability of paedomorphic populations of *E. tynerensis* in the western Ozark Plateau (Bonett & Chippindale 2006). The Slunger Creek valley supports two metamorphic, stream-dwelling plethodontids (*E. multiplicata* and *Desmognathus brimleyorum*). Dominant vegetation at the type locality is second growth mixed pine-hardwood forest composed of *Pinus echinata* and several hardwoods (*Acer*, *Celtis*, *Cornus*, *Liquidambar, Ulmus*, and *Quercus*).

**Life history and ecology**. Most aspects of *E. subfluvicola* life history are derived from observations in captivity, and reproductive status has been determined by examining the gonads through the transparent venter. Enlargement of testes was first observed in the field in February, and reached maximum size in the lab by late June and July. Like related *E. multiplicata* and *E. tynerensis*, *E. subfluvicola* typically has single lobed testes (Sever 1974; MAS pers. obs.), but one individual possessed multiple lobes (Fig. 9b). Mature or maturing egg follicles have only once been observed in the field (late November), however, follicles started maturing in the lab (at 20ºC) in June and were robust by August. We have not yet found definitive juveniles of *E. subfluvicola* in the field, which suggests that early development (<32mm SVL) may be primarily subterranean.

Coinciding with peak testicular size, the five largest captive males began to show signs of metamorphosis (i.e. gill reduction, changes in head morphology and coloration). For four individuals, the process took over 2.5 months, and metamorphosis was never completed before the animals died. *Eurycea multiplicata* can complete metamorphosis in less than one month after initiation under similar conditions (MAS pers. obs.). Two of the partially metamorphosed *E. subfluvicola* showed remodeling of the hyobranchial apparatus towards the adult form (Alberch *et al.* 1985). This includes the loss of larval epibranchials, and formation of the adult epibranchial, and the reshaping of the ceratohyal, basibranchials, and ceratobranchials. Adult maxillary morphology (Wilder 1925; Wake 1966) started to form in three specimens. The small external gills were also never completely absorbed. These individuals demonstrate that facultative metamorphosis might be possible in this species, however, given that four of the five large males died during this process (with the 5$^{th}$ individual still undergoing metamorphosis 5-February 2014), indicates that metamorphosis appears to be a grave event, as seen in some other paedomorphic salamanders that have naturally or experimentally metamorphosed (Dundee 1957, 1962; Brandon 1976). It is notable that partial metamorphosis only occurred in the largest, most reproductive males (based on testicular examination), while females showed no signs of metamorphosis (n=13). We hypothesize that high levels of endogenous androgens may induce metamorphosis of male *E. subfluvicola* in captivity. Studies have shown that testosterone, but not estradiol, can increase the rate of metamorphosis in other salamander species (Norris *et al.* 1973). Surface activity of *Eurycea subfluvicola* is primarily nocturnal, with individuals leaving the cover of rocks or leaf litter during the day. During periods without surface flow, individuals of *E. subfluvicola* presumably follow the water table using interstitial spaces in the streambed gravel. The subterranean ecology of *E. subfluvicola* in the hyporheic zone is completely unknown.

## Discussion

We present life history, genetic and morphological evidence for the description of the first paedomorphic plethodontid from the Ouachita Mountains. *Eurycea subfluvicola* is the most divergent paedomorphic salamander described in the past 70 years; since the descriptions of *E. tynerensis* (compared to *E. multiplicata*; Moore & Hughes 1939) and *E. wallacei* (compared to *E. bislineata*; Carr 1939). Other more recently described paedomorphic salamanders are much more closely related to their sister taxa/clades, which in almost all cases are also paedomorphic (e.g. Netting & Goin 1942; Neill 1964; Chippindale *et al.* 2000; Hillis *et al.* 2001; for divergence times see Bonett *et al.* 2013, 2014). *Eurycea subfluvicola* also represents an additional independent instance of paedomorphosis within Spelerpini (now with 18 paedomorphic species; AmphibiaWeb 2014). Interestingly, *E. subfluvicola* appears to share similar ecology with paedomorphic populations of *E. tynerensis* from

the western Ozark Plateau (Bonett & Chippindale 2006). Like *E. tynerensis*, *E. subfluvicola* appear to undergo seasonal movements into the streambed when the surface streams recede. Paedomorphosis in *E. subfluvicola* seems to have enabled access to an otherwise unexploited subterranean niche, and may be important in maintaining reproductive isolation with syntopic *E. multiplicata*.

Over the last decade we sampled *E. multiplicata* from over 100 locations throughout the Ouachita Mountains, but often only metamorphic adults were collected and sequenced. Our fortuitous discovery of a highly divergent larval form in 2011 prompted intensive examination and sequencing of both larvae and adults in and around Lake Catherine State Park, which allowed us to delineate the new species. *Eurycea subfluvicola* represents an example of how species that primarily differ by an ontogenetic shifts can mask species diversity. This phenomenon may be more likely in organisms which display complex life histories, where deviant adult forms can masquerade as different developmental stages of related species. This discovery highlights how much biodiversity may still remain hidden, even in well studied areas.

*Eurycea subfluvicola* currently has one of the smallest, if not the smallest, known distribution of any North American salamander (AmphibiaWeb 2014). Surveys up and downstream of these sites, and several nearby streams, have yet to produce additional specimens, despite the presence of adult and larval *E. multiplicata*. It is possible that previously sampled *E. multiplicata* localities across the Ouachita Mountains also contain *E. subfluvicola*, since collection of larval forms was not emphasized. Regardless, *E. subfluvicola* is of immediate conservation concern, given this species' current known distribution. Fortunately, the known distribution of *E. subfluvicola* is within a protected site (Lake Catherine State Park). To ensure the long-term conservation of this unique species we recommend the following actions: 1) conduct additional surveys to further delineate the distribution; 2) establish conservation measures (as applicable) to preserve known sites; and 3) captive propagation efforts to ensure reserve stock in case of wild population extirpation.

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